

## From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION  
(PCT Rule 61.2)

To:

United States Patent and Trademark  
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(Box PCT)  
Crystal Plaza 2  
Washington, DC 20231  
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 12 July 1999 (12.07.99)	
International application No. PCT/CA98/01016	Applicant's or agent's file reference DH/11468.92
International filing date (day/month/year) 29 October 1998 (29.10.98)	Priority date (day/month/year) 29 October 1997 (29.10.97)
Applicant SEGUELA, Philippe et al	

1. The designated Office is hereby notified of its election made:

in the demand filed with the International Preliminary Examining Authority on:

25 May 1999 (25.05.99)

in a notice effecting later election filed with the International Bureau on:

\_\_\_\_\_

2. The election

was

was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Authorized officer

S. Mafla

Facsimile No.: (41-22) 740.14.35

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Form PCT/IB/331 (July 1992)

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# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference DH/11168.92	<b>FOR FURTHER ACTION</b>		See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/CA98/01016	International filing date (day/month/year) 29/10/1998	Priority date (day/month/year) 29/10/1997	
International Patent Classification (IPC) or national classification and IPC C12N15/12			
Applicant MCGILL UNIVERSITY et al.			

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 5 sheets, including this cover sheet.

This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I     Basis of the report
- II     Priority
- III     Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV     Lack of unity of invention
- V     Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI     Certain documents cited
- VII     Certain defects in the international application
- VIII     Certain observations on the international application

Date of submission of the demand 25/05/1999	Date of completion of this report 08.09.99
Name and mailing address of the international preliminary examining authority:   European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer  Huber, A Telephone No. +49 89 2399 8173



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/CA98/01016

**I. Basis of the report**

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

**Description, pages:**

1-14                   as originally filed

**Claims, No.:**

1-15                   as originally filed

**Drawings, sheets:**

1/11-11/11           as originally filed

2. The amendments have resulted in the cancellation of:

- the description,       pages:  
 the claims,           Nos.:  
 the drawings,         sheets:

3.  This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/CA98/01016

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

**1. Statement**

Novelty (N)	Yes:	Claims 1-15
	No:	Claims
Inventive step (IS)	Yes:	Claims 1-15
	No:	Claims
Industrial applicability (IA)	Yes:	Claims 1-15
	No:	Claims

**2. Citations and explanations**

**see separate sheet**

**VI. Certain documents cited**

**1. Certain published documents (Rule 70.10)**

**and / or**

**2. Non-written disclosures (Rule 70.9)**

**see separate sheet**

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/CA98/01016

**SECTION V**

1. The present application relates to the isolation and cloning of a DNA encoding a human proton-gated cation channel (hASIC3) and the protein encoded thereby.
2. Reference is made to the following document:

D1: WALDMANN R ET AL: 'Molecular cloning of a non-inactivating proton-gated Na<sup>+</sup> channel specific for sensory neurons' JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 34, 22 August 1997, pages 20975-20978, XP002080467 cited in the application
3. D1 discloses the molecular cloning of a non-inactivating proton-gated Na<sup>+</sup> channel from rat specific for sensory neurons (DRASIC). The amino acid sequence of DRASIC, which apparently has the highest homology of known cation channels with that of the present application, is 83.5% identical with that of hASIC3.
4. The subject-matter of Claim 1, requiring that the proton-gated cation channel has at least 85% identity with SEQ ID No.1, is considered novel in view of D1 (Art. 33(2) PCT).

In view of the prior art, the problem to be solved by present application can be seen in the provision of a further (human) proton-gated cation channel. The problem has been solved by the isolation of hASIC3 by screening the dbEST database with probes corresponding to a conserved protein motif present in all known members of the degenerin/ENaC/ASIC family.

There is, however, no teaching in the prior art which would directly and unambiguously lead the skilled person to the isolation of the claimed protein or the DNA encoding it. From D1 it cannot be derived in an obvious manner that a human homologue to DRASIC exists which could be isolated by using a conserved amino acid stretch present in DRASIC and other known ion channels. Therefore an inventive step can be acknowledged for the subject-matter of Claims

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/CA98/01016

1-15 (Art. 33(3) PCT).

**SECTION VI -----**

**Certain published documents (Rule 70.10)**

Application No Patent No	Publication date (day/month/year)	Filing date (day/month/year)	Priority date (valid claim) (day/month/year)
WO-A-98/54316	03.12.1998	15.05.1998	30.05.1997

Said document has been published and filed after the claimed priority date of present application. It does therefore not constitute prior art in the sense of Rule 64(1)(b) PCT. Since it claims a priority date which is earlier than that of present application, it may, however, become of relevance for the novelty of the claimed subject-matter during regional phase examination.

## PATENT COOPERATION TREATY

PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>DH/11168.92</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/CA 98/01016</b>	International filing date (day/month/year) <b>29/10/1998</b>	(Earliest) Priority Date (day/month/year) <b>29/10/1997</b>
Applicant <b>MCGILL UNIVERSITY et al.</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

It is also accompanied by a copy of each prior art document cited in this report.

**1. Basis of the report**

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
  - the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :
  - contained in the international application in written form.
  - filed together with the international application in computer readable form.
  - furnished subsequently to this Authority in written form.
  - furnished subsequently to this Authority in computer readable form.
  - the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
  - the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished
- 2.  Certain claims were found unsearchable (See Box I).
- 3.  Unity of invention is lacking (see Box II).

4. With regard to the **title**,

- the text is approved as submitted by the applicant.
- the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

- the text is approved as submitted by the applicant.
- the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

- as suggested by the applicant.
- because the applicant failed to suggest a figure.
- because this figure better characterizes the invention.

5

None of the figures.

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/CA 98/01016

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:  
Claims 1-6, 13, 15 encompass uses *in vivo* and can be considered methods of treatment or diagnosis. The search has been limited to the protein claimed *per se* and its alleged effects and uses *in vitro*.
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

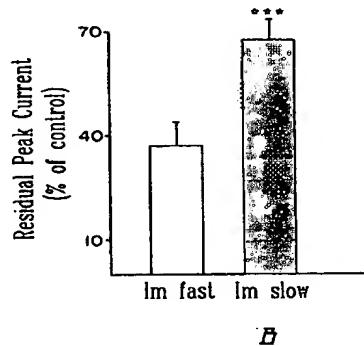
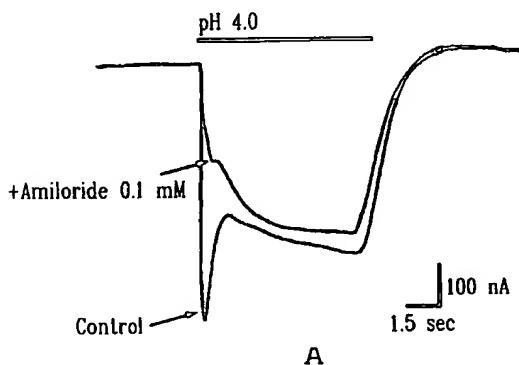
- The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY-(PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/12, 5/10, C07K 14/705, G01N 33/566, A61K 38/17</b>		A1	(11) International Publication Number: <b>WO 99/21981</b> (43) International Publication Date: <b>6 May 1999 (06.05.99)</b>
(21) International Application Number: <b>PCT/CA98/01016</b>		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: <b>29 October 1998 (29.10.98)</b>			
(30) Priority Data: <b>2,219,713 29 October 1997 (29.10.97) CA</b>			
(71) Applicant ( <i>for all designated States except US</i> ): <b>McGILL UNIVERSITY [CA/CA]; 3550 University Street, Montréal, Québec H3A 2A7 (CA).</b>			
(72) Inventors; and		Published	
(75) Inventors/Applicants ( <i>for US only</i> ): <b>SEGUELA, Philippe [CA/CA]; 890 Davaar, Outremont, Québec H2V 4B5 (CA). BABINSKI, Kazimierz [CA/CA]; 364 Berkley Circle, Dorval, Québec H9S 1H4 (CA).</b>		<i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(74) Agents: <b>DUBUC, Jean, H. et al.; Goudreau Gage Dubuc &amp; Martineau Walker, The Stock Exchange Tower, Suite 3400, 800 Place-Victoria, P.O. Box 242, Montréal, Québec H4Z 1E9 (CA).</b>			

(54) Title: DNA ENCODING A HUMAN PROTON-GATED ION CHANNEL AND USES THEREOF



## (57) Abstract

Small changes of extracellular pH activate depolarizing inward currents in most nociceptive neurons. It has been recently proposed that acid sensitivity of sensory as well as central neurons is mediated by a family of proton-gated cation channels structurally related to *C. elegans* degenerins and mammalian epithelial sodium channels. We describe here the molecular cloning of a novel human proton receptor, hASIC3, a 531 amino acid-long subunit homologous to rat DRASIC. Expression of homomeric hASIC3 channels in *Xenopus* oocytes generated biphasic inward currents elicited at pH < 5, providing the first functional evidence of a human proton-gated ion channel. Contrary to the DRASIC current phenotype, the fast desensitizing early component and the slow sustained late component differed both by their cationic selectivity and by their response to the antagonist amiloride, but not by their pH sensitivity (pH50= 3.66 vs 3.82). Using RT-PCR and mRNA blot hybridization, we detected hASIC3 mRNA in sensory ganglia, brain and many internal tissues including lung and testis, so hASIC3 gene expression was not restricted to peripheral sensory neurons. These functional and anatomical data strongly suggest that hASIC3 plays a major role in persistent proton-induced currents occurring in physiological and pathological conditions of pH changes, likely through a tissue-specific heteropolymerization with other members of the proton-gated channel family.

**FOR THE PURPOSES OF INFORMATION ONLY**

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**TITLE OF THE INVENTION**

DNA ENCODING A HUMAN PROTON-GATED ION-CHANNEL AND USES THEREOF

**5      FIELD OF INVENTION**

In mammals, the pH of the extracellular compartment, including interstitial fluids and blood, is strictly regulated and maintained at a constant value of 7.4. Acid sensing is a specific kind of chemoreception that plays a critical role in the detection of nociceptive pH imbalances occurring, for example, in conditions of cramps, trauma, inflammation or hypoxia (Lindahl, 1974). In mammals, a population of small-diameter primary sensory neurons in the dorsal root ganglia and trigeminal ganglia express specialized pH-sensitive surface receptors activated by increase of extracellular proton concentration (Bevan and Yeats, 1991). Acid sensitivity of sensory as well as central neurons is mediated by a family of proton-gated cation channels structurally related to *C. elegans* degenerins and mammalian epithelial sodium channels. This invention relates to these Acid Sensing Ion Channels (ASIC), particularly to a non-inactivating proton-gated channel, named hASIC3, its association with other channel subunits and uses thereof.

**20     BACKGROUND OF INVENTION**

Tissue acidosis is associated with a number of painful physiological (e.g. cramps) and pathological conditions (e.g. inflammation, intermittent claudication, myocardial infarction). Experimentally, similar painful events can be reproduced by infusing low pH solutions into skin or muscle. Furthermore, the prolonged intradermal infusion of low pH solutions can mimic the characteristic hyperalgesia of chronic pain. To further characterize the effects of protons and their relation to pain, low pH solutions were applied to patch-clamped central and peripheral sensory neurons. Inward currents were induced when pH was dropped to acidic values, providing evidence for the existence of proton-activated ion channels. Several types of native currents were observed in sensory neurons from rat and human trigeminal and dorsal root ganglia:

- rapidly inactivating currents;
- non-inactivating currents; and
- biphasic currents displaying a rapidly inactivating current followed by a non-inactivating current.

Other differences regarding ion selectivities were also reported. These results suggested the existence of several proton-gated ion channels. The prolonged pain induced by tissue acidification is most likely associated with a non-inactivating proton-gated ion channel.

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### **Cloned prot n-gated i n channels**

Three classes of mammalian proton-gated cation channels have recently been cloned in rat and named « ASIC » for Acid Sensing Ion Channels. Sequence analysis identifies them as members of the DEG/ENaC superfamily of ion channels. The putative membrane topology of ASIC receptors predicts two transmembrane spanning domains with both N- and C-termini in the intracellular compartment, as shown for the epithelial sodium channels (ENaC). The published ASIC receptors are described below:

- 5 1) **ASIC1A** (first reported as ASIC), displays a rapidly inactivating current with a pH<sub>50</sub> of 6.2 (Waldmann R. et al., *Nature* 1997; **386**: 173-7). N.B.: pH<sub>50</sub> indicates the pH at which peak inward current equals half the maximal value.
- 10 2) **ASIC1B** (initially reported as ASIC-β), is a splice variant of ASIC1A where the first 185 amino acids of ASIC1A are replaced by a distinct new sequence of 172 amino acids. ASIC1B shows similar current kinetics and pH<sub>50</sub> as those observed for ASIC1A (Chen C-C et al., *Proc Natl Acad Sci* 1998; **95**: 10240-5).
- 15 3) **ASIC2A** (first reported as MDEG, then MDEG1), displays a slowly inactivating current with a pH<sub>50</sub> of 4.05 (Waldmann R. et al., *J Biol Chem* 1996; **271**: 10433-6).
- 20 4) **ASIC2B** (first reported as MDEG2) is a splice variant of ASIC2A where the first 185 amino acids are replaced by a distinct new sequence of 236 amino acids (Lingueglia E. et al., *J Biol Chem* 1997; **272**: 29778-83). When expressed in heterologous expression systems, ASIC2B does not appear to be activated by protons.
- 25 5) **DRASIC**, which displays a biphasic current where the rapidly inactivating component has a pH<sub>50</sub> of 6.5 and the non-inactivating component a pH<sub>50</sub> of 3.5 (Waldmann R. et al., *J Biol Chem* 1997; **272**: 20975-8).

### **25 Tissue distribution of cloned proton-gated ion channels**

ASIC1A and ASIC2B mRNAs are present in both brain and sensory neurons. ASIC2A mRNA is detected in the central nervous system but is absent in sensory neurons. ASIC1B and DRASIC are exclusively expressed in sensory neurons, predominantly in small diameter neurons. The different ASIC subunits in brain (ASIC1A, ASIC2A, ASIC2B) as well as the ASICs in sensory neurons (ASIC1B, ASIC2B and DRASIC) appear to be coexpressed in the same neurons (Waldmann R. et al., *Curr Opinion Neurobiol* 1998; **8**: 418-24).

### **Homo- and heteromultimeric assembly of proton-gated channel subunits**

35 Ion channels are multimeric complexes, which result from the association of several identical (homopolymeric) and/or different (heteropolymeric) channel subunits. Except for ASIC2B, all ASIC subunits associate into homomultimeric complexes and yield the functional channels described above. In addition, certain ASIC subunits have been shown to form functional heteromultimeric channels with distinctive properties. Indeed, ASIC2B, which by itself does not give rise to a proton-gated channel, modifies

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the chann I characteristics of ASIC2A and of DRASIC when coexpressed with either on or the other. The homomultimeric ASIC2A channel has a single exponential inactivation profile and is highly selective for sodium. When coexpressed with ASIC2B, the inactivation kinetics become biphasic with a slowly inactivating component that poorly discriminates between sodium ( $\text{Na}^+$ ) and potassium ( $\text{K}^+$ ) ions. Similarly, when DRASIC was coexpressed with ASIC2B, the sustained sodium-selective current of DRASIC became cation non-selective ( $\text{pNa}^+ = \text{pK}^+$ ) (Lingueglia E. et al., *J Biol Chem* 1997; 272: 29778-83).

***Proton-gated channels are related to degenerins and mechanosensation***

As mentioned above, ASICs belong to the DEG/ENaC superfamily of receptors, which also includes epithelial sodium channels (ENaC) involved in sodium homeostasis, the FMRFamide peptide-activated channel FaNaC from *Helix aspersa*, involved in neurotransmission, the ATP-gated cation channels, also involved in neurotransmission, as well as the degenerins of *Caenorhabditis elegans*, involved in mechanotransduction. As mentioned above, involvement of proton-gated ion channels in nociception seems likely. However, since many of the ASIC subunits are also expressed elsewhere than in sensory neurons, other functions than nociception must also be considered. In addition to their noxious effects, protons might play important roles as neurotransmitters as implied by reports of neuronal activity in response to fluctuations of pH. However, it still remains to be proven that sufficient pH changes can occur to activate ASIC channels with low  $\text{pH}_{50}$ , such as ASIC2A or ASIC2A+ASIC2B channels. Alternatively, ASIC channels might also be activated by other ligands, such as neuropeptides and neurotransmitters, or by mechanical energy, as suggested by their sequence homology with the other members of the DEG/ENaC superfamily.

25

**SUMMARY OF INVENTION**

The object of the present invention is to provide the primary structure, functional characterization and tissue distribution of a human non-inactivating amiloride-sensitive proton-gated ion channel, designed herein as hASIC3.

30 hASIC3 is not the orthologue of rat DRASIC as supported by the following evidence:

1) hASIC3 has 83% sequence identity with rat DRASIC while human and rat ASIC1A orthologues show an identity of 97.7% and human and rat ASIC2A orthologues show an identity of 99%. (Garcia-Anoveros J. et al., *Proc Natl Acad Sci* 1997; 94: 1459-64).

35 2) Tissue distribution of hASIC3 is widespread throughout the body while rat DRASIC mRNA is restricted to sensory neurons (compare Fig. 6 and Waldmann R. et al., *J Biol Chem* 1997; 272: 20975-8)

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3) hASIC3 and rat DRASIC biphasic currents display different properties. In the following discussion «fast component» will refer to the rapidly inactivating current and «slow component» will refer to the sustained current, which follows the fast component:

- 5      ■ The fast and slow component of hASIC3 have similar pH<sub>50</sub>s (3.66 and 3.82, respectively), while rat DRASIC has different pH<sub>50</sub>s for the fast and slow components (6.5 versus 3.5, respectively) (compare Fig. 3 and Waldmann et al., *J Biol Chem* 1997; 272: 20975-8).
- 10     ■ The slow component of hASIC3 is inhibited by amiloride while the slow component of rat DRASIC is potentiated by amiloride (compare Fig. 5A and 5B and Waldmann et al., *J Biol Chem* 1997; 272: 20975-8).
- 15     ■ Reversal potentials for the fast and slow components of hASIC3 differed by 15 mV ( $E_{rev}$  fast= +33 mV and  $E_{rev}$  slow = +48 mV), while both fast and slow components of rat DRASIC have the same  $E_{rev}$  of +32 mV. (compare Fig. 4 and Waldmann et al., *J Biol Chem* 1997; 272: 20975-8).

15     Another aim of the present invention is to provide a DNA sequence encoding a novel subtype of a human non-inactivating amiloride-sensitive proton-gated ion channel, hASIC3 and derivatives thereof.

20     In accordance with the present invention there is provided an isolated nucleic acid molecule, which consists essentially of the nucleotide sequence depicted in SEQ ID No. 1, and derivatives thereof.

The isolated nucleic acid molecule of the present invention encodes a peptide consisting essentially of the amino acid sequence listed in SEQ ID No.: 2, and derivatives thereof.

25     RNA encoding the hASIC3 receptor, transcribable from DNA in accordance with the present invention (SEQ ID No.: 1) and substantially free from other RNAs, also forms part of the invention, and may be useful for a number of purposes including hybridization studies, *in vitro* translation as well as translation in appropriate *in vivo* systems such as *Xenopus* oocytes.

30     The present invention also relates to complete and/or partial complementary and/or antisense nucleotide sequences corresponding to the nucleotide sequence listed in SEQ ID No. 1.

35     In accordance with the present invention, there is provided a vector, preferably an expression vector, selected from the group consisting of plasmid, phage, retrovirus, baculovirus, adenovirus and integration elements, which include the isolated nucleic acid molecule of the present invention.

The present invention also relates to host cells transformed or transfected with a vector as described above. Host cells may be prokaryotic or eukaryotic and include mammalian cells (such as COS, CHO cells and human embryonic kidney cells, HEK293), insect cells, yeasts (such as *Saccharomyces cerevisiae*) and bacteria (such

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as *Escherichia coli*). Host cells will either transiently express hASIC3 and/or derivatives thereof, as in the case of COS cells, or be stably transfected with a vector carrying the hASIC3 sequence and/or derivatives thereof. A CHO cell line or any other cell line that stably expresses hASIC3 and/or any derivatives thereof can be used for electrophysiological, calcium-influx, ion-imaging, ligand-binding, affinity purification, immunoprecipitation, western blotting and immunoblotting studies. Host cells which do not express the receptor may still be useful as cloning hosts.

Another object of the present invention is to provide a method to isolate a ligand that will bind the hASIC3 protein, or any derivative thereof.

A hASIC3 and/or a derivative thereof prepared by recombinant DNA technology in accordance with the invention has a number of uses, either *in situ* in the membrane of the expression host or in *in vitro* systems. In particular, the receptor can be used as a screen for ligands and/or compounds useful in a variety of human (or other animal) diseases and conditions, such as pain, inflammation, ischemia and neurodegenerative disorders. Ligands refers to any chemical or biological entity that binds to any intracellular and/or extracellular region or portion of the hASIC3 and/or its derivatives. Such ligands include compounds present in combinatorial chemical libraries, peptid phage display libraries, extracts containing unknown compounds (for example plant extracts, marine life extracts, toxins, venoms), as well as biological molecules such as polyclonal and/or monoclonal antibodies, neurotransmitters, peptides or inorganic ions and metals.

For example, in accordance with the present invention there is provided a method of using the isolated nucleic acid molecule listed in SEQ ID No. 1, or a sequence which hybridizes under stringent conditions to the sequence listed in SEQ ID No. 1, to produce a peptide consisting essentially of the amino acid sequence listed in SEQ ID No. 2, which comprises the steps of:

- a) transforming a host with a DNA sequence capable of encoding the peptide
- b) incubating the host under conditions which allow the protein sequence to be expressed and
- c) isolating by all means the protein from the host.

Recording or imaging the activity of the protein from the host can be performed to confirm or monitor the activity of the protein. This method can be reiterated, this time, with two or more DNA sequences encoding two or more different proteins, which results in the obtention of heteropolymeric channels.

Derivatives of hASIC3 include functional and/or structural variants as described below:

- Derivatives of hASIC3 include molecules whose sequence differs from the hASIC3 sequence by a modification and/or substitution and/or insertion and/or deletion of one or several amino acid residues as long as this modification

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and/or substitution and/or insertion and/or deletion does not modify the functional and/or structural properties of the hASIC3 channel, mainly the activation by protons. The amino acid sequence of derivatives must be at least 85% or greater identical to the amino acid sequence of hASIC3. Such  
5 derivatives can be synthesized and/or analysed by a person skilled in the art following established techniques.

■ Derivatives of hASIC3 also include molecules whose sequence differs from the hASIC3 sequence by a modification and/or substitution and/or insertion and/or deletion of one or several amino acid residues even if this modification and/or  
10 substitution and/or insertion and/or deletion does modify the functional and/or structural properties of the hASIC3 channel, rendering it non-or differently responsive to protons.

Examples of derivatives are hASIC3-like channel subunits corresponding to the partial nucleotide sequences (Expressed Sequence Tag available from public dbest  
15 databases, such as EST # AI024055, AA628357, AA448259, AA449322, hASIC3 channel subunits epitope-tagged on N-terminus and/or C-terminus, as well as hASIC3 channel subunit where the first 150-200 amino acids were substituted by a new and different amino acid sequence, in a similar fashion as reported for ASIC1A and ASIC1B, and ASIC2A and ASIC2B.  
20

The present invention relates to the association of hASIC3 and derivatives thereof with other channel subunits such as the proton-gated ion channel subunits and derivatives thereof (for example ASIC1A, ASIC1B, ASIC2A, ASIC2B), or other channel subunits related to the DEG/ENaC superfamily of receptors, such as alpha-, beta-, gamma-, delta-ENaC subunits or the P2x ATP-gated ion channel subunits. Such  
25 associations include interactions between subunits of different species, but the preferred associations are between subunits from the same species.

An example of heteromeric association between hASIC3 and rat P2x2 is given herein below. The resulting new channel is a novel proton-gated ion channel, which displays higher sensitivity to pH. Indeed, when hASIC3 and P2x2 are coinjected into  
30 Xenopus oocytes, inward currents can be activated with slight acidification to pH 6.5, while the homomeric hASIC3 requires a pH of 4.0. This different pharmacological property proves that hASIC3 and P2x2 physically associated to generate a new ion channel.

35 **DESCRIPTION OF INVENTION**

This invention will be described by way of specific embodiments, examples and figures, which purpose is to illustrate the invention rather than to limit its scope.

**BRIEF DESCRIPTION OF THE DRAWINGS**

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**Figure 1.** Nucleotide and predicted amino acid sequence of hASIC3 cDNA (SEQ ID No. 1 and No. 2, respectively) (A), deposited in Genbank database under the accession number AF057711. Two stretches of 20-34 amino acids corresponding to potential transmembrane domains, identified in Kyte-Doolittle hydrophobicity plot, are highlighted. Consensus phosphorylation sites in intracellular domains and N-glycosylation sites in extracellular domain are indicated by circled and boxed residues, respectively. Homology (in %) of hASIC3 (B) and phylogenetic relationships (C) with known members of the human ASIC/ENaC family. Protein dendrogram generated using UPGMA algorithm (Geneworks 2.5.1, Oxford Molecular Group).

**Figure 2.** Biphasic current phenotype of homomeric hASIC3 channels: effects of increasing rates of pH change. Oocytes were clamped at -70 mV and continuously perfused with Ringer's buffer containing 10 mM HEPES at pH 7.6. pH was then dropped to 4.0 for 10 sec with increasing pH gradients obtained by raising the buffer capacity differential between control and test buffers: A. pH 7.6 to pH 4.0 in 10 mM HEPES; B. pH 7.6 in 5 mM to pH 4.0 in 10 mM HEPES; and C. pH 7.6 in 5 mM to pH 4.0 in 20 mM HEPES. Controls done with the test buffers at pH 7.6 did not activate the hASIC3 channel. Oocytes injected with injection buffer alone showed no inward currents. The amplitude of the early but not the late component, and thus the ratio of early/late peak currents, appears to be very sensitive to the speed at which the pH drops from normal to acid pH values.

**Figure 3.** Dose-response curves of pH activation of hASIC3 currents. Dose-response curves were constructed in 20 mM HEPES buffered at different pH values from 6.0 to 3.0. Peak currents of fast and slow currents were analysed with the four parameter logistic equation and the partial F test for statistical comparison. Each point represents mean  $\pm$  SEM from this typical experiment. Apart from the maximal response, no other significant difference was observed between both curves.

**Figure 4.** Current-voltage (I/V) relationship of the fast and slow hASIC3 currents. Recordings were done in Ringer's buffer containing (in mM): NaCl 115, KCl 2.5, CaCl<sub>2</sub> 1.8 and HEPES 5, pH 7.6. The I/V relationship was established by measuring peak currents of both fast and slow responses to pH 4.0 (applied 1 sec after voltage step) at different membrane potentials, after subtracting background currents recorded without pH applications (A). Peak current values were plotted (B) and reversal potential estimated from linear (slow current) and nonlinear (fast current) regression analysis. Panels A and B represent a typical experiment where reversal potentials were 33.4 mV and 44.8 mV respectively for the fast and slow currents.

**Figure 5.** Differential sensitivity of the fast and slow hASIC3 currents to amiloride. hASIC3 currents were activated by pH 4.0 in the presence and absence of 100  $\mu$ M amiloride (A). Inhibition by amiloride was much stronger on the fast than the sustained current. Data in B represent mean  $\pm$  SEM ( $n=17$ ) of residual peak currents during

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amiloride application for the fast (37.2 ± 6.4%) and sustained (72.0 ± 3.8%) components expressed as percent of controls. Statistical significance (P) was evaluated by unpaired two-tailed t-test (\*\* P<0.001).

5 **Figure 6.** Distribution of hASIC3 mRNA in normal human tissues. High stringency hybridization of radiolabeled hASIC3 cDNA on human polyA+ RNA isolated from brain, spinal cord, internal tissues as well as from 17-28 weeks fetal tissues was quantitated by densitometric analysis in phosphorimaging. Amounts of polyA+ RNA target were normalized across tissues to allow direct comparisons of transcription levels (see Materials and Methods for details).

10 **Figure 7.** High levels of transcription of hASIC3 gene in sensory ganglia enriched in nociceptive neurons. Localization of hASIC3 and reporter G3PDH mRNAs in human trigeminal ganglia (TG), cerebellum (CB) and lung (L) using RT-PCR amplification with specific exact match primers (see Materials and Methods section for details).

15 **Figure 8.** Illustration of a recording of non-inactivating cation current induced by strong acid (pH 4.0) in *Xenopus* oocytes injected with hASIC3 clone alone in pcDNA3 vector.

15 **Figure 9.** Illustration of a recording of non-inactivating cation current induced by weak acid (pH 6.5) in *Xenopus* oocytes coinjected with hASIC3 clone and rat P2x2 clone, both in pcDNA3 vector.

20 **EXAMPLE 1: Characterization of a human non-inactivating proton-gated ion channel, hASIC3**

#### Introduction

25 Acid sensing is a specific kind of chemoreception that plays a critical role in the detection of nociceptive pH imbalances occurring in conditions of cramps, trauma, inflammation and hypoxia (Lindahl, 1974). In mammals, a population of small-diameter primary sensory neurons in the dorsal root ganglia and trigeminal ganglia express specialized pH-sensitive surface receptors activated by increase of extracellular proton concentration (Bevan and Yeats, 1991). Native electrophysiological responses of sensory neurons to applications of pH 5.8-6.5 are characterized by a fast desensitizing inward current followed by a slow sustained current (Krishtal and Pidoplichko, 1981).

30 Elucidating the native molecular composition of proton sensors in human sensory neurons will be an important step in the rational development of a novel class of analgesics. A family of genes coding for neuronal proton-gated channels subunits has been recently discovered (Garcia-Anoveros et al., 1997; Waldmann et al., 1997). Heterologously expressed amiloride-sensitive homomeric rat ASIC (Acid Sensing Ion Channel) (Waldmann et al., 1997) responds to small pH changes by a fast desensitizing sodium-selective current, while MDEG1 (Mammalian DEGenerin 1) (Waldmann et al., 1996) and DRASIC (Dorsal Root ganglia ASIC) (Waldmann et al., 1997) require drastic pH changes to gate desensitizing and biphasic currents, respectively. ASIC and MDEG1 can associate together to generate a heteromeric

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channel activated at low pH (<5) with unique kinetics and ionic selectivities (Bassilana et al., 1997). A neuronal splicing variant of MDEG1 was shown to modulate DRASIC biophysical properties by heteromeric association (Lingueglia et al., 1997). These proton-gated channels share a putative two-transmembrane domain topology and 5 co-localization in small diameter capsaicin-sensitive sensory neurons with P2X ATP-gated channels (North, 1997). From their sequence, they belong to an expanding gene superfamily including mammalian epithelial sodium channels (Canessa et al., 1994), pickpocket (PPK) and ripped pocket (RPK) subunits from *Drosophila* (Adams et al., 1998), degenerins of *C. elegans* (Corey and Garcia-Anoveros, 1996), and the 10 FMRFamide-gated channel of *Helix aspersa* (Lingueglia et al., 1995). Despite their potential importance in monitoring pH changes in central nervous system and sensory pathways, human proton receptor genes have not yet been functionally characterized. We report here for the first time the heterologous expression of a human proton-gated 15 channel, as well as significant inter-species differences observed both in functional properties and regional distributions of acid sensors.

#### **Material and Methods**

##### **Molecular cloning**

Using the tblastn algorithm, virtual screening of the dbEST database of NCBI (Lennon et al., 1996) with probes corresponding to the protein motif 20 LXFP AVTLCN XNXRXS, conserved in all known members of the degenerin/ENaC/ASIC family, led to the identification of human EST sequences encoding a novel member of the proton sensor gene family (Genbank accession numbers AA449579 and AA429417). The clone tagged by 5' EST AA449579 and by 3' EST AA449322 from a total fetus cDNA library was sequenced on both strands 25 using walking primers and an ALF DNA sequencer (Pharmacia-LKB). Full-length hASIC3 was directionally subcloned into unique EcoRI and NotI sites of eukaryotic vector pcDNA3 (Invitrogen) for CMV promoter-driven heterologous expression in *Xenopus* oocytes.

##### **Electrophysiology in *Xenopus* oocytes**

30 Oocytes surgically removed from adult *Xenopus laevis* were treated for 2 h at room temperature with type II collagenase (Gibco-BRL) in Barth's solution under constant agitation. Selected oocytes at stage IV-V were defolliculated manually before nuclear micro-injections (Bertrand et al., 1991) of 5 ng of hASIC3 in pcDNA3 vector. After 2-4 days of expression at 19°C in Barth's solution containing 50µg/ml gentamycin, 35 currents were recorded in the two-electrode voltage clamp configuration using an OC-725B amplifier (Warner Instruments). Whole cell currents were acquired and digitized at 500 Hz on a Macintosh IIci computer with an A/D NB-MIO16XL interface (National Instruments) then recorded traces were post-filtered at 100 Hz in Axograph (Axon Instruments). Agonist, amiloride and wash solutions were prepared in a modified

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Ringer's solution containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl<sub>2</sub> in 5-20 mM HEPES (Sigma) buffer adjusted with NaOH or HCl at pH 2 to 8 and applied on oocytes by constant perfusion (10-12 ml/min) at room temperature. Mean values ± SEM corresponded to measurements from a minimum of 5 oocytes.

5      **RT-PCR and mRNA dot blot hybridization**

Total RNA from post-mortem samples of normal human trigeminal ganglia were isolated using Trizol reagent (Gibco-BRL), then 1 µg was subjected to random-primed reverse transcription using Superscript (Gibco-BRL). Around 100 ng of RT-cDNA was used as template for PCR with Expand DNA polymerase (Boehringer-Mannheim).

10     Specific hASIC3 primers TCAGTGGCCACCTTCCTCTA (forward) and ACAGTCCAGCAGCATGTCATC (reverse) were used to amplify the region corresponding to nucleotides 175-513 (Fig.IA). After initial template denaturation of 2 min at 94°C, thermal cycles consisted of 45 sec at 94°C, 45 sec at 55°C and 2 min at 72°C for 30 cycles. Molecular identity and homogeneity of PCR products were checked  
15     by sizing and specific restriction patterns. Initial sample loading was checked by co-amplification of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) housekeeping mRNA. RNA samples not subjected to reverse transcription but PCR amplified in identical conditions provided our negative controls.

Known amounts of human polyA+ RNA (89-514 ng), isolated from various fetal  
20    and adult normal tissues and normalized for the transcription levels of several housekeeping genes (Clontech), were dot-blotted and probed with the [32P]-labeled EcoRI-XbaI fragment of hASIC3 cDNA at high stringency (final elution at 65°C in 0.3 X SSC buffer for 10 min). After exposure for 16 hours, hybridization signals were acquired and quantitated using a Storm phosphorimager (Molecular Dynamics), then  
25    analyzed in densitometry with ImageQuant software (Molecular Dynamics).

**Results**

**Primary structure of hASIC3 channel subunit**

Sequence analysis of the 1.7 kb-long hASIC3 polyA+ mRNA revealed an open reading frame encoding 531 amino acids (Fig. IA), with initiation of translation at the proximal Met codon located at position nt. 22. The predicted molecular weight of 59 kDa for the immature protein was confirmed by *in vitro* translation (data not shown). According to the current topological model based on primary structure analysis and biochemical tests, a large domain of 365 amino acids faces the extracellular side of the plasma membrane (Canessa et al., 1994). In this extracellular domain, a total of 15 cysteine residues are highly conserved in the ASIC family, with the exception of Cys267 being absent in human BNaC1 (hASIC2) only. Two potential sites for Asn-linked glycosylation, Asn175 and Asn398, are located in this Cysteine-rich loop. Consensus sites for phosphorylation by cas in kinase II (Ser5) and by protein kinase C (Ser39, Ser478, Ser493, Ser521) are found in the intracellular N-terminal domain of

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hASIC3 as well as in the intracellular C-terminal domain (Fig. 1A). The hASIC3 subunit displays 83% of identity with rat DRASIC subunit at the amino acid level, 48% with human BNaC2 (hASIC1) and 47% with BNaC1 (hASIC2) (Fig. 1B). Therefore hASIC3 belongs to the proton-gated channel family, itself a branch of the degenerin/ENaC/FMRFamide-gated channel phylogenetic tree (Fig. 1C).

#### 5 Functional and pharmacological properties of homomeric hASIC3 channels

When heterogeneously expressed in *Xenopus* oocytes, hASIC3 subunits assemble into functional homomeric channels activated by low extracellular pH (Fig. 2A). Rapid changes of extracellular pH (Fig. 2B-C) revealed a biphasic response. This unique 10 phenotype was characterized by a fast and rapidly desensitizing current followed by a slow and sustained current which returned to baseline only upon return to physiological pH. The relative amplitude of the fast current appeared dependent on the slope of the pH gradient applied (Fig. 2A-C). However, we found the pH sensitivity of the two hASIC3-mediated currents to be almost identical with a pH<sub>50</sub> of 3.66 ± 0.06 15 (fast) vs 3.82 ± 0.04 (slow) (Fig. 3). The positive cooperativity reflected in the dose-response curve profile, nH<sub>fast</sub>=1.57 ± 0.3 and nH<sub>slow</sub>=1.55 ± 0.17, indicated that at least two protonations on two subunits are required in order to gate the cation channel. To study possible differences of ionic selectivity between fast and slow components, we performed a current-voltage relationship at pH 4.0 in normal Ringer. 20 The peak amplitude of the fast component displayed some voltage-dependence from its slight inward rectification, while the slow and sustained component was ohmic in the range from -70 to + 70 mv (Fig. 4B). Furthermore, although both reversal potentials were greater than 30 mv as expected for channels conducting mainly sodium, we measured a  $E_{rev}$  = +15 ± 3.2 mv ( $p < 0.01$ ) between the fast (+32.9 ± 4.4 mv) and 25 the slow component (+48.2 ± 4.8 mv) (Fig. 4A-B). These two phases of proton-induced hASIC3 current differed also by their sensitivity to the antagonist amiloride. Co-application of 100 μM amiloride with pH 4.0 in conditions of biphasic response demonstrated a more efficient blockade of the fast (62.8 ± 6.5%) than of the slow current (28.7 ± 4.6%) by amiloride (Fig. SAB).

#### 30 Central and peripheral distribution of hASIC3 gene expression

As a rough index of anatomical distribution and mRNA abundance, we noticed several cDNAs encoding hASIC3 in total fetus and testis cDNA libraries represented in the dbEST database. Results obtained in RNA hybridization at high stringency confirmed that the hASIC3 gene is transcribed in a wide spectrum of internal organs 35 as well as in the central nervous system (Fig. 6). In the adult stage, hASIC3 transcripts were detected in lung, lymph nodes, kidney, pituitary, heart and testis as well as in brain and spinal cord. A developmental up-regulation of hASIC3 gene expression was apparent when comparing fetal vs adult mRNA levels in lung and kidney (Fig. 6). Thus hASIC3 subunit expression is not restricted exclusively to sensory ganglia as is rat

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DRASIC, explaining our decision to use a chronological nomenclature that is distribution-independent. The potentially important function of non-inactivating proton-gated channels in nociceptive sensory neurons was nevertheless confirmed by the detection of high levels of ASIC3 mRNA in adult human trigeminal ganglia using 5 RT-PCR (Fig. 7).

**EXAMPLE 2: Heteromultimeric channel composed of hASIC3 and rat P2x2**

To demonstrate the possibility of heteromultimeric association between hASIC3 and/or derivatives thereof and other ion channel subunits, we have coexpressed the hASIC3 and rat P2x2 constructs in *Xenopus* oocytes to verify if the presence of an 10 ATP-gated ion channel subunit can modify hASIC3 channel properties. P2X2 displays important similarities with hASIC3, such as overall membrane topology (two transmembrane spanning domains, cysteine rich extracellular domain), sensitivity to pH (response to ATP of the P2X2 homomultimeric channel is strongly potentiated by acid pH), and tissue distribution (both hASIC3 and P2X2 are colocalized in sensory 15 neurons).

There is shown in Fig. 8 the recording of non-inactivating cationic current induced by strong acid (pH 4.0) in *Xenopus* oocytes injected with the hASIC3 clone alone in pcDNA3 vector. These data demonstrate that hASIC3 alone can associate in functional homomeric cation channels.

20 There is shown in Fig. 9 the recording of non-inactivating cation current induced by weak acid (pH 6.5) in *Xenopus* oocytes coinjected with hASIC3 clone and rat P2x2 clone, both in pcDNA3 vector. These data demonstrate that the co-expression of hASIC3 and rat P2x2 changes the pH sensitivity of homomeric hASIC3 or leads to the formation of heteromultimeric pH-sensitive channels.

25 While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice 30 within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

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**Claims:**

1. The use of a molecule having the amino acid sequence of SEQ ID NO:1 or a variant having at least 85% identity therewith, as a proton-gated cation channel.
2. The use as defined in C1, wherein said molecule forms a homopolymeric channel.
3. The use as defined in C1, in combination with another cation channel sub-unit which forms a heteropolymeric channel with said molecule.
4. The use as defined in C3, wherein said other cation channel belongs to the degenerin/ENaC channel superfamily.
5. The use as defined in C3, wherein said other cation channel is a P2X ATP-gated channel.
6. The use as defined in C5, wherein said other cation channel sub-unit is P2X2 ATP-gated channel sub-unit.
7. A recombinant host cell comprising a first and a second DNA sequences respectively capable of expressing the molecule and the other cation channel as defined in any one of claims 3 to 6.
8. The use of the recombinant host cell as defined in C7, in the making of a composition or a kit for screening compounds useful as proton-gated cation channel ligands.
9. The use of the recombinant host cell as defined in C7 for screening compounds useful as proton-gated cation channel ligands.
10. The use of a nucleic acid encoding a molecule as defined in any one of C1 to 6, in the making of a composition or a kit for screening compounds useful as proton-gated cation channel ligands.
11. The use of a nucleic acid encoding a molecule as defined in any one of C1 to 6, for screening compounds useful as proton-gated cation channel ligands.
12. The use of a molecule as defined in any one of C1 to 6, in the making of a composition or a kit for screening compounds useful as proton-gated cation channel ligands.
13. The use of a molecule as defined in any one of C1 to 6, for screening compounds useful as proton-gated cation channel ligands.
14. A composition or kit for screening compounds useful as proton-gated cation channel ligands, which comprises a molecule as defined in any one of C1 to 6, a nucleic acid encoding said molecule or a recombinant host capable of expressing said molecule, and any suitable auxiliary component.
15. The use as defined in any one of C8 to 13, or the composition or kit as defined in C14, wherein said ligands are analgesics.

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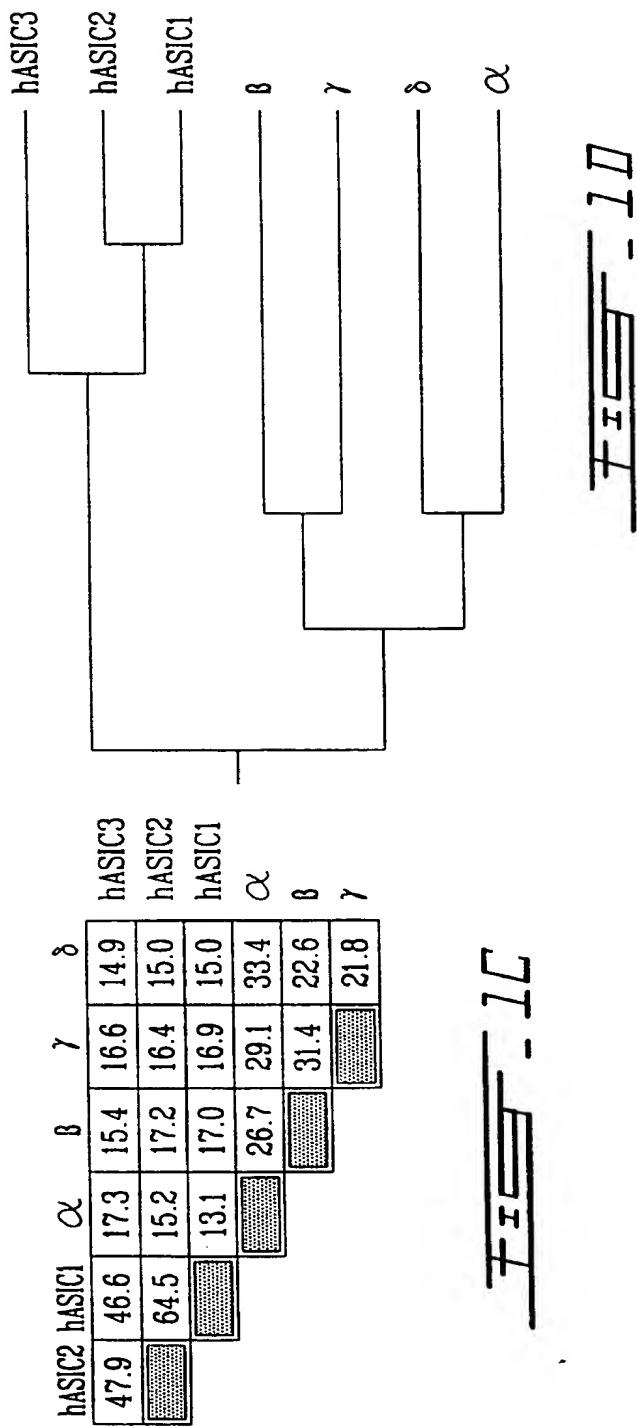
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V F A S N C S M H G L G H V F G P G S L (S) L R R G	43
ATGTCGGCAGGCCGTTGCTCAGTGGCACCTTACCGGTGGCTGAGAGGGTGGCTACTACAGG	225
M W A A V V L S V A T F L Y Q V A E R V R Y Y R	68
GAGTTCCACCAGACTGCCCTGGATGAGCGAGAAAGCCACCGGCTCGTCTGGCTTCCGGCTACCCCTGTGCCAAC	300
E F H H Q T A L D E R E S H R L V F P A V T L C N	93
ATCAAACCCACTGGCCGGCTCGGCCATAACGCCAACGACCTGCACTGGGCTCTGGCTGGCTGGAT	375
I N P L R R S R L T P N D L H W A G S A L L G L D	118
CCCGCAGAGCACGCCGGCTTCCCTGGCGCCGGCCCTGGGGCCGGCCGGCTGCACCCGGCCGGCTCATGCCAGTCCCAC	450
P A E H A A F L R A L G R P P A P P G F M P S P T	143
TTTGACATGGCCAACCTATGCCCGTGTGGCACTCCCTGGATGACATGCTGGACTGCTGGCTTCCGGTGGC	525
F D M A Q L Y A R A G H S L D D M L L D C R F R G	168
CAACCTTGTGGCCCTGAGAACTCACCACGATCTCACCCGGATGGGAAGTGTACACATTAACTCTGGCGCT	600
Q P C G P E [N] F T T I F T R M G K C Y T F N S G A	193
GATGGGGCAGAGCTGCTCACCACTACTAGGGTGGCATGGCAATGGGCTGGACATCATGCTGGCAGCTGCAGCAG	675
D G A E L L T T R G G M G N G L D I M L D V Q Q	218
GAGGAATATCTACCTGTGGAGGGACAATGAGGAGACCCCGTTGAGGTGGGGATCCGAGTCACAGC	750
E E Y L P V W R D N E E T P F E V G I R V Q I H S	243
CAGGAGGGAGCCCCATCATCGATCAGCTGGGGCTTGGGGCTACCGACCTTGTGTTCTGCCAG	825
Q E E P P I I D Q L G V S P G Y Q T F V S C Q	268
CAGCAGCTGAGCTTCCCTGCCACCGGCCCTGGCAGTCAGGATCTGAACCCCCAACTATGAGCCA	900

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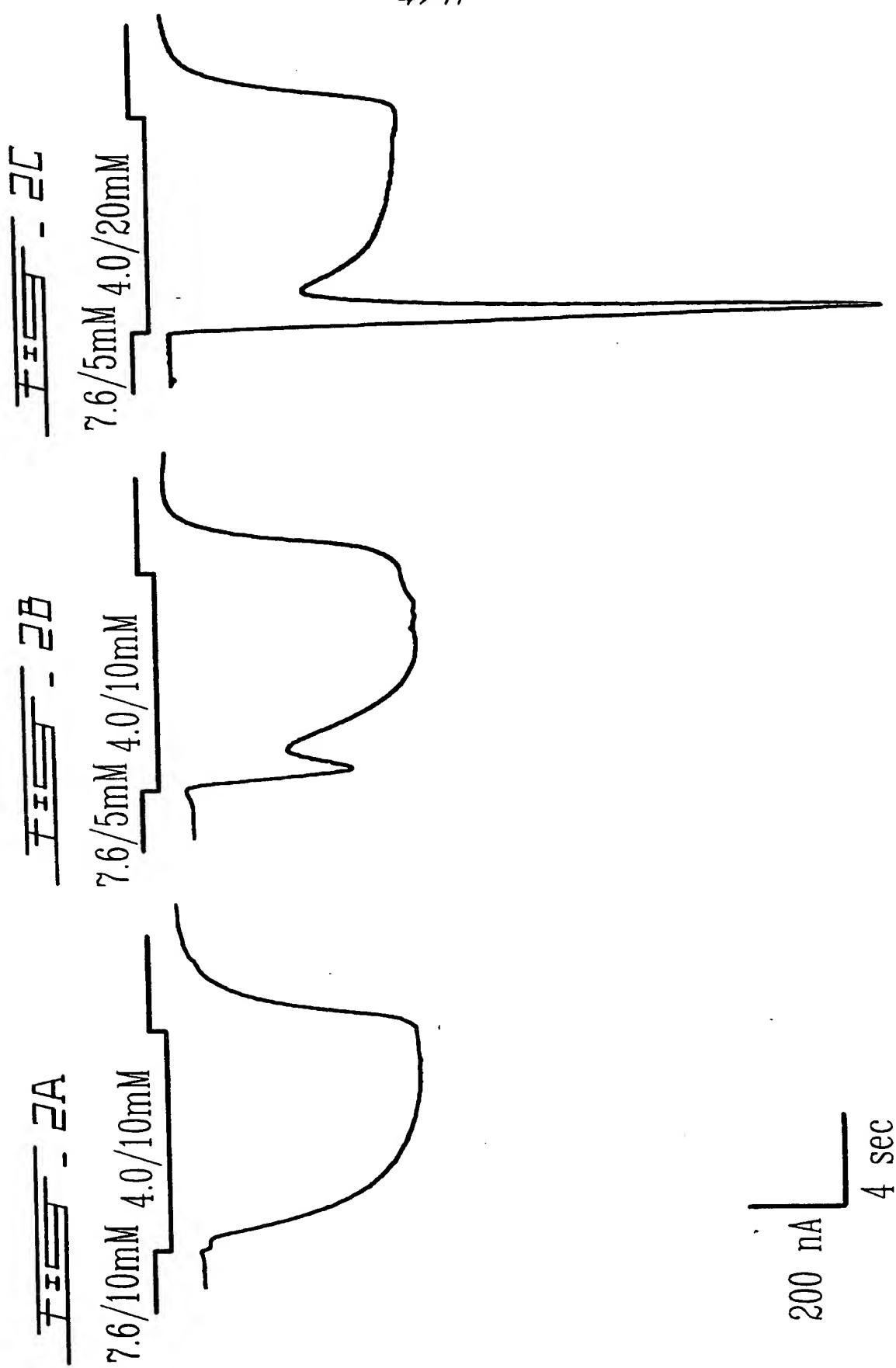
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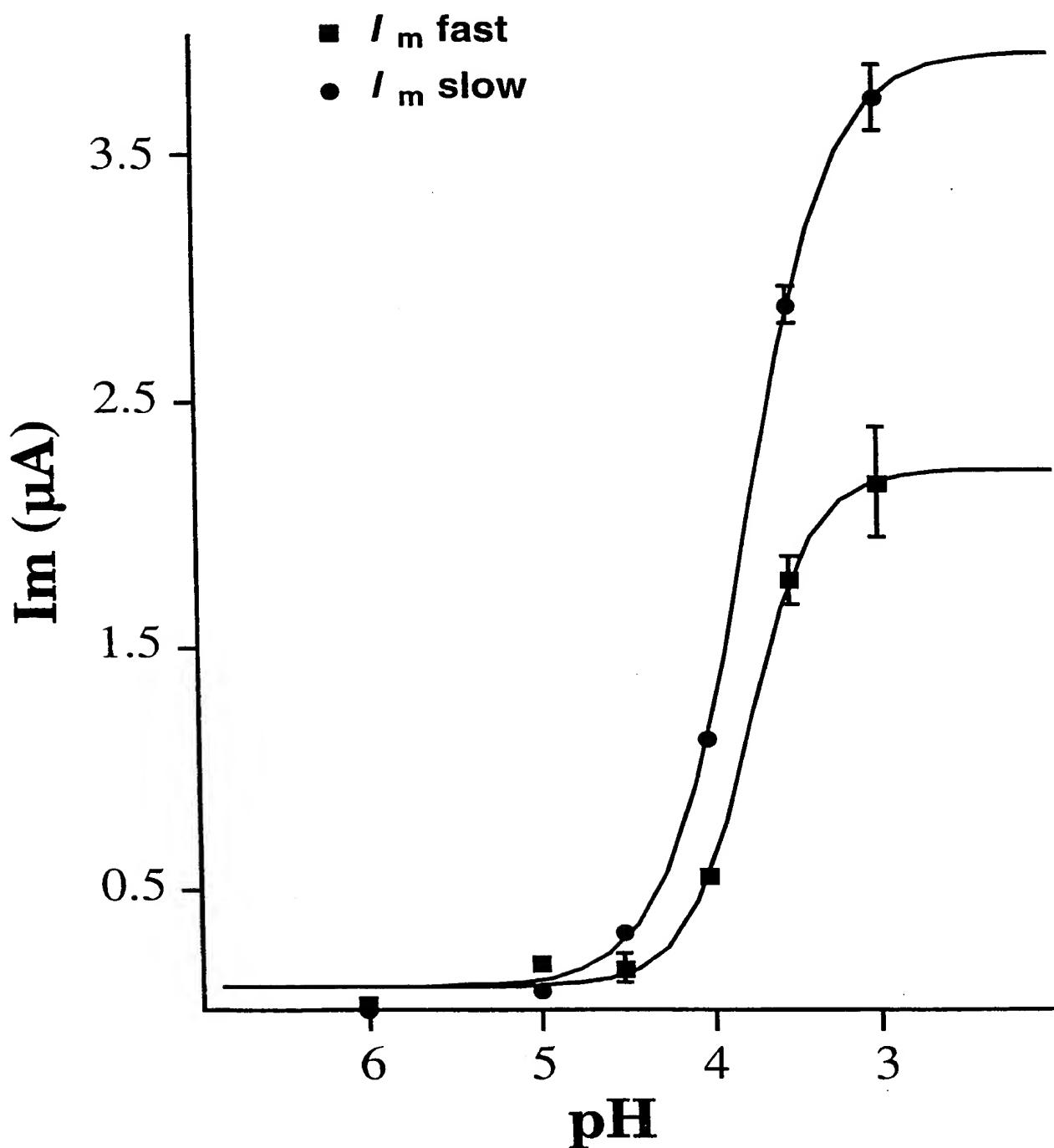


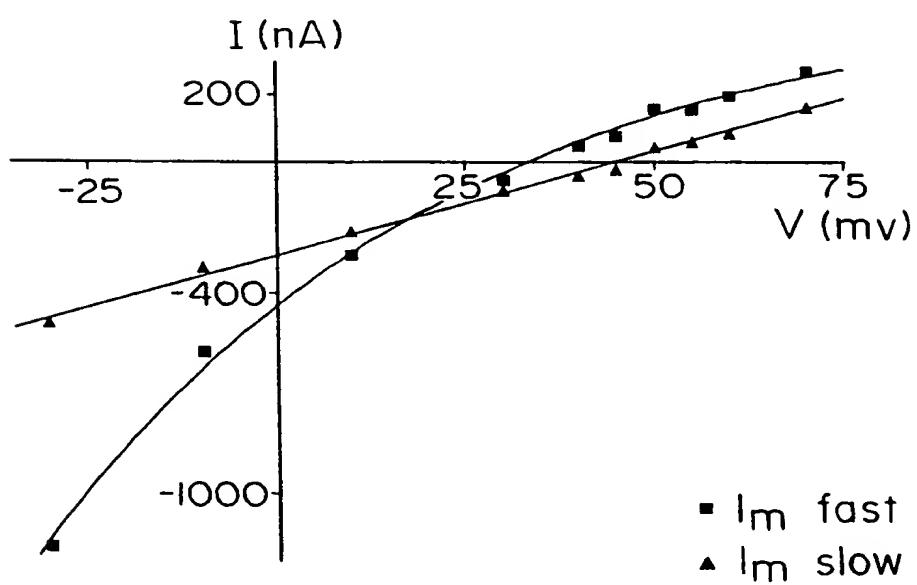
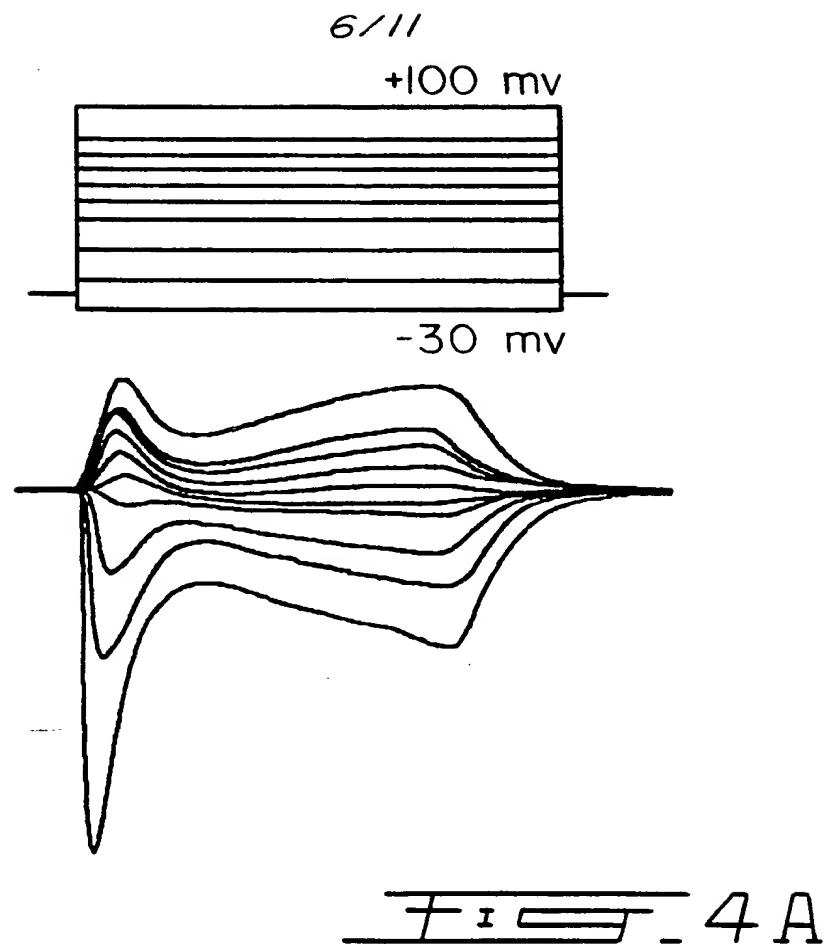
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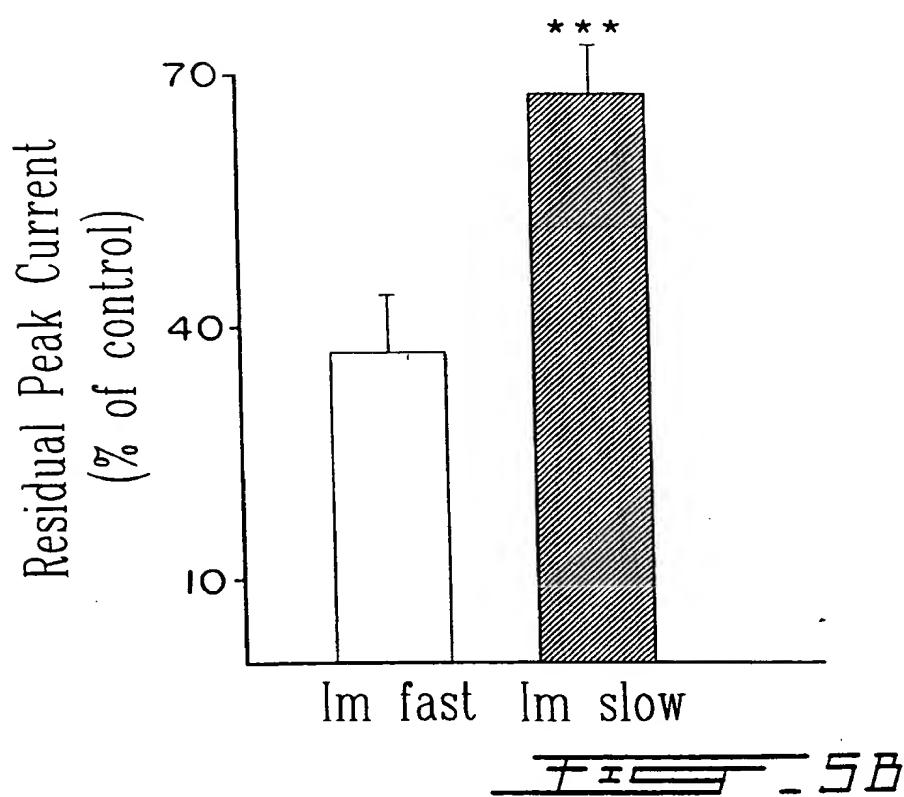
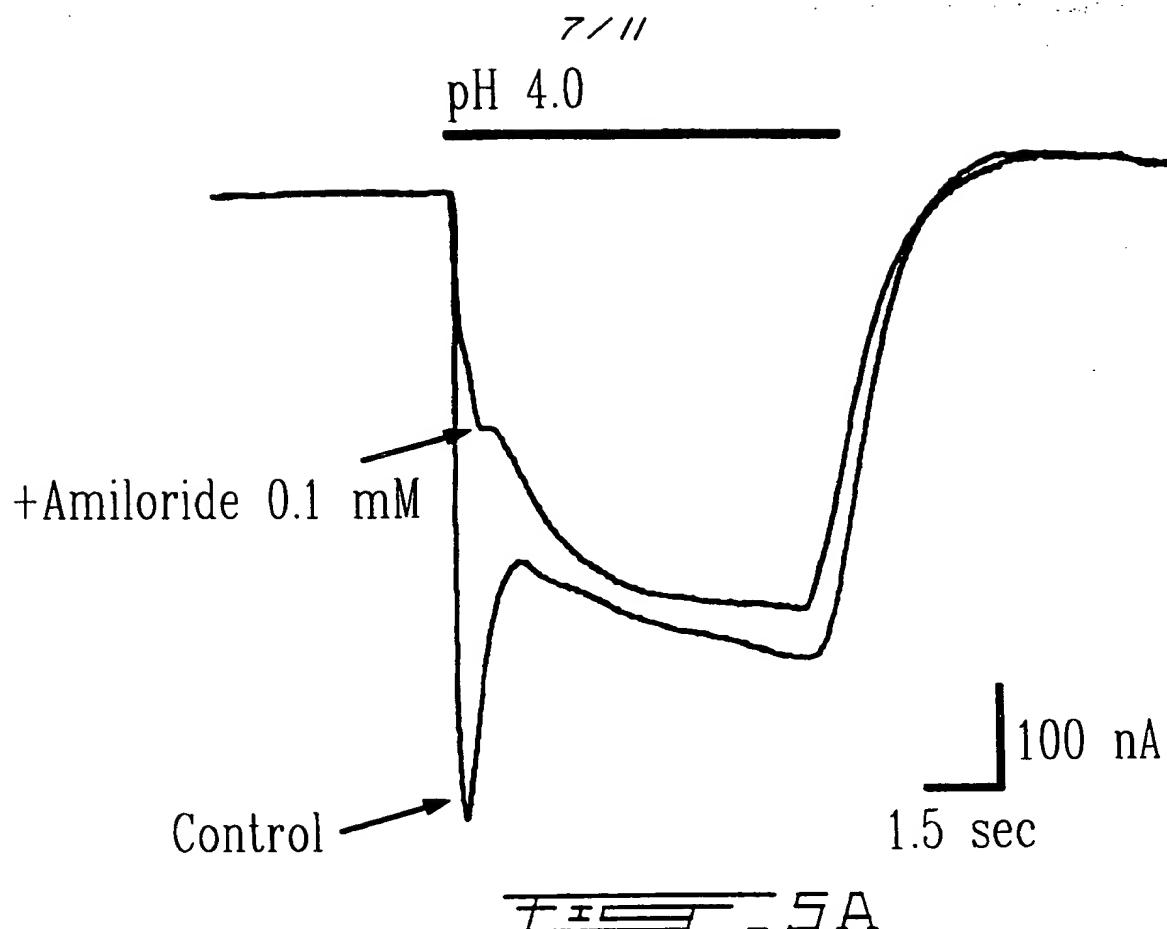
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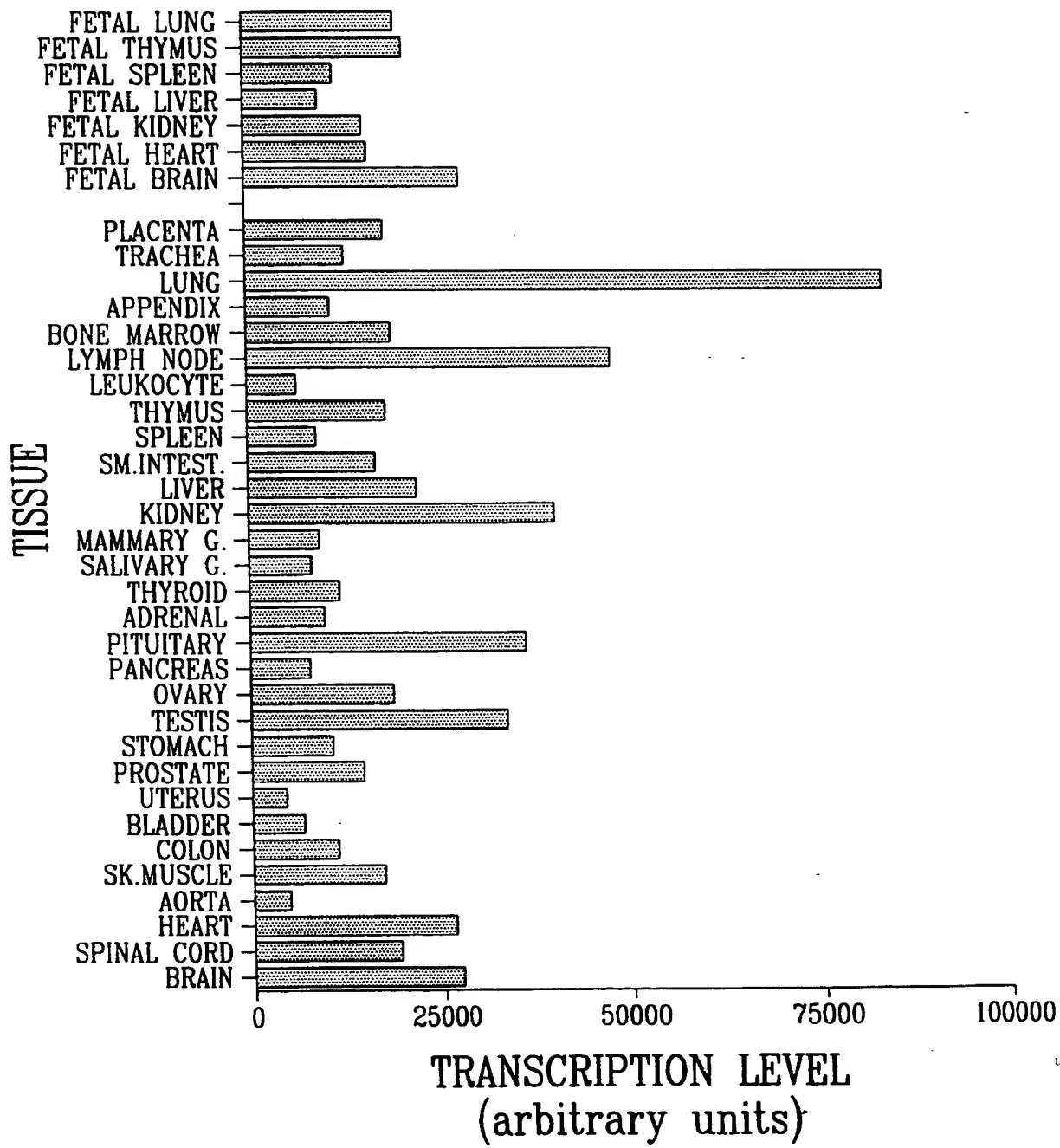
~~FIGURE 3~~



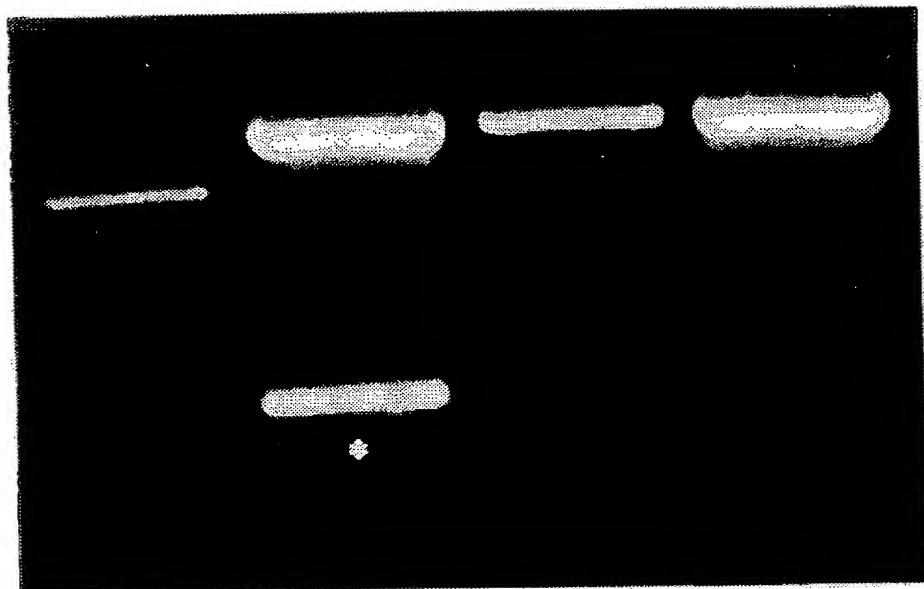
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Markers

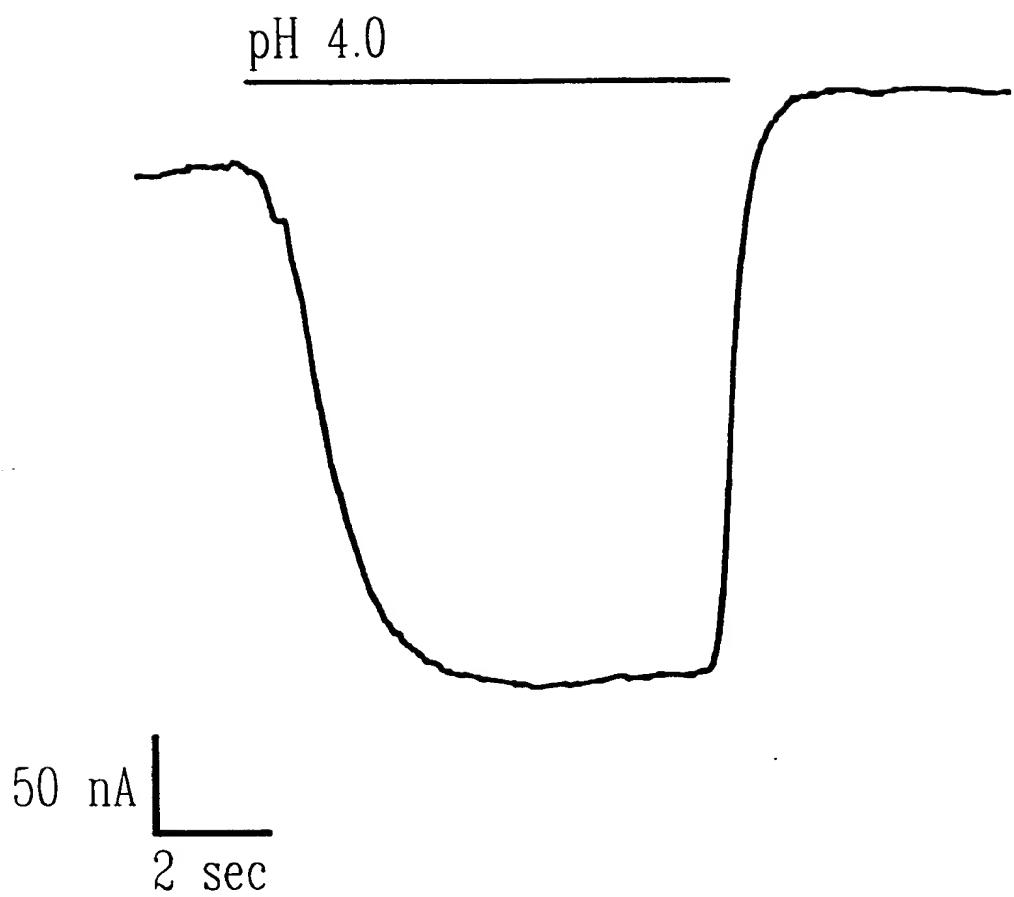
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pH 6.5

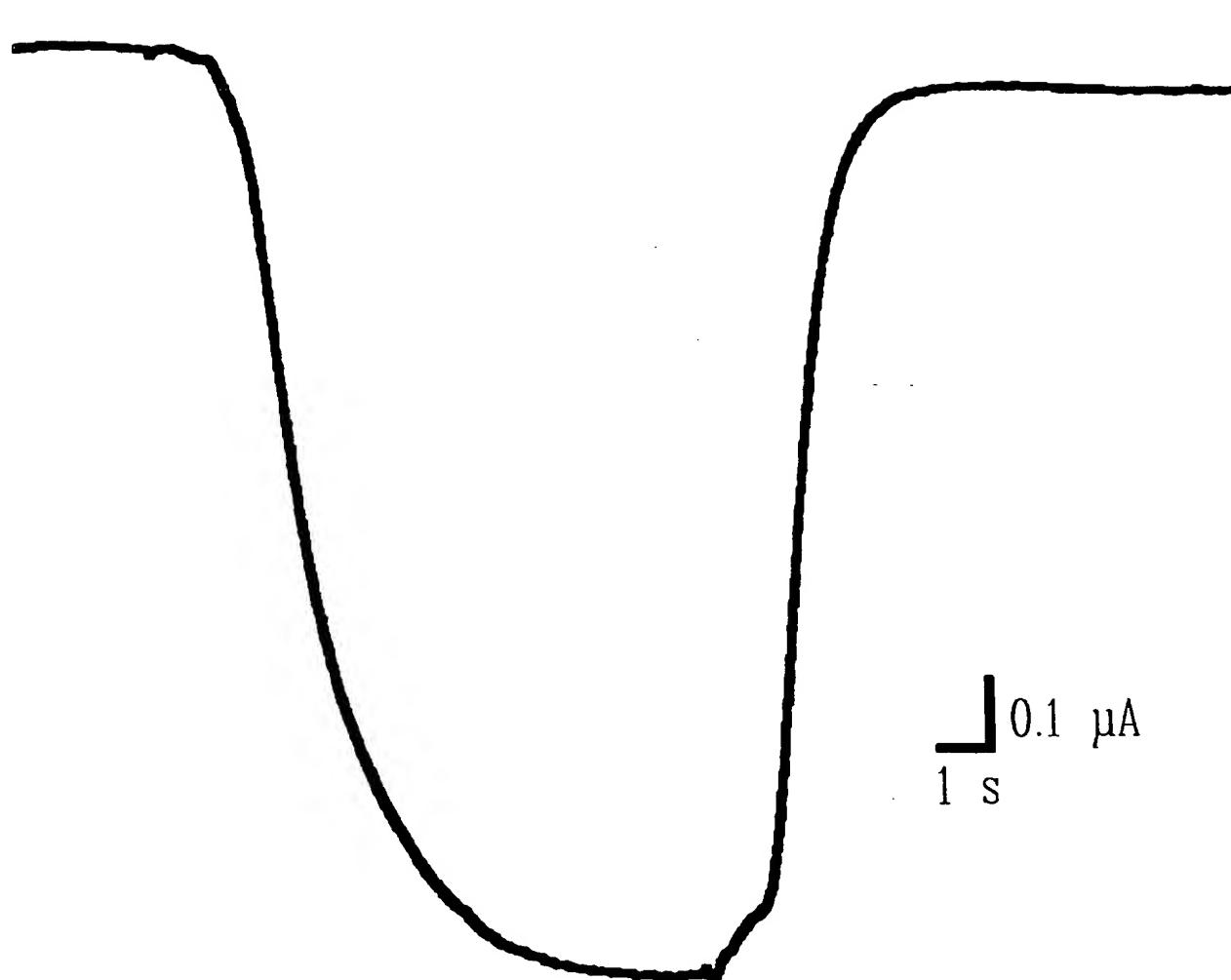


FIG. 9

## INTERNATIO

## SEARCH REPORT

Inte Application No

PCT/CA 98/01016

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C12N5/10

C07K14/705

G01N33/566

A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WALDMANN R ET AL: "Molecular cloning of a non-inactivating proton-gated Na <sup>+</sup> channel specific for sensory neurons" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 34, 22 August 1997, pages 20975-20978, XP002080467 cited in the application see the whole document ---	1-15
A	WO 97 01577 A (UNIV LONDON) 16 January 1997 see abstract see claims 1-20 ----	1-15 -/-

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

## ° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

6 April 1999

16/04/1999

## Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

Galli, I

## INTERNATIONAL SEARCH REPORT

Inter.	Application No.
PCT/CA 98/01016	

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LEWIS C. ET AL.: "Coexpression of P2X2 and P2X3 receptor subunits can account for ATP-gated currents in sensory neurons." NATURE, vol. 377, 5 October 1995, pages 432-435, XP002098923 see the whole document ---	4-6
A	COREY D P ET AL: "MECHANOSENSATION AND THE DEG/ENAC ION CHANNELS" SCIENCE, vol. 273, no. 5273, 19 July 1996, page 323/324 XP002051360 cited in the application see the whole document ---	4
P, X	DATABASE GENBANK Accession No. AF057711, 7 October 1998 BABINSKI K. ET AL.: "H. sapiens proton-gated cation channel subunit (ASIC3)" XP002098924 see the whole document ---	1-15
P, X	WO 98 35034 A (HEURTEAUX CATHERINE ;CHAMPIGNY GUY (FR); LINGUEGLIA ERIC (FR); WAL) 13 August 1998 see abstract see seq. ID 5 see claims 1-25 ---	1-15
E	WO 98 54316 A (GRAHAM DAVID ;RENARD STEPHANE (FR); SYNTHELABO (FR); BESNARD FRANC) 3 December 1998 see abstract see seq. ID 6 see claims 1-28 -----	1-15

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA 98/01016

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:  
Claims 1-6, 13, 15 encompass uses *in vivo* and can be considered methods of treatment or diagnosis. The search has been limited to the protein claimed *per se* and its alleged effects and uses *in vitro*.
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

- The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

Inter Application No

PCT/CA 98/01016

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9701577 A	16-01-1997	AU CA EP	6234596 A 2221294 A 0835261 A	30-01-1997 16-01-1997 15-04-1998
WO 9835034 A	13-08-1998	FR FR	2759372 A 2759373 A	14-08-1998 14-08-1998
WO 9854316 A	03-12-1998	EP	0884386 A	16-12-1998